ANALYTICAL CHROMATOGRAPHY OF MYOSIN ON HYDROXYAPATITE

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SUMMARY

Preparations of rabbit myosin have been subjected to chromatographic fractionation on columns of hydroxyapatite at pH 6.8 with potassium phosphate buffers containing KCl. The best resolution is obtained with a stepwise elution procedure, utilizing phosphate concentrations of 0.16, 0.23, and 0.4 M, all in the presence of 0.4 M KCl. The ionic strengths of these buffers cover the range 0.7 to 1.4. The chromatograms obtained are very consistent, as are the elution volumes at a given phosphate concentration, and the elution volumes are strong functions of phosphate concentration. The fractions obtained are well separated, and clear-cut information is obtained on the presence in the preparation of proteins other than myosin and on the extent of aggregation of the myosin. Since the recovery of adenosine triphosphatase activity is low the procedure described cannot be used for preparative purposes.

INTRODUCTION

Two of the most powerful tools for analytical and preparative fractionation and purification of proteins, *viz.* adsorption chromatography and electrophoresis, have been of limited use in work with myosin. This results primarily from the insolubility of this protein, at pH values near neutrality, at ionic strengths less than 0.25 (ref. 1), a range which is generally most useful in applying the techniques in question. Furthermore, myosin solutions contain the protein in non-monomeric form even at ionic strengths greater than 0.25 (ref. 2), the amount in the monomeric form increasing with increasing ionic strength.

The difficulty can be circumvented by the use of either (1) an adsorbent which is effective even in solvents of relatively high ionic strength (the higher the better), or (2) a solvent which is exceptional in that myosin is soluble in it at relatively low ionic strength. Recent work in this laboratory has made use of both of these approaches. The latter alternative has been applied to electrophoresis of myosin. The former alternative constitutes the subject matter of this paper.

Chromatography on hydroxyapatite is strongly dependent on the concentration of phosphates, *e.g.* orthophosphate, but is otherwise virtually independent of ionic strength. Since KCl–phosphate solvents of sufficient ionic strength (greater than 0.3)

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are favored solvents for myosin, chromatography on hydroxyapatite with such solvents appeared to offer a reasonable chance of success. Particularly appealing was the possibility of elution with solvents of high ionic strength ($\gg$ 0.7), constant pH and KCl concentration, and increasing phosphate concentration, either in a gradient or in a stepwise system. These predictions were found to be correct, and the results of their application are reported herewith.

MATERIALS AND METHODS

Preparation of myosin

All preparative operations, as well as chromatography, were carried out at 2–5°. Phosphate buffers were made with the potassium salts.

Myosin was extracted, usually from the longissimus dorsi muscles of the rabbit, with Guba–Straub solution (0.3 M KCl; potassium phosphate buffer, 0.15 M in phosphate; pH 6.59 $\pm$ 0.05; temperature, 3°). The protein was rendered insoluble by addition of water in two stages[3] until the conductivity of the solution was equal to that of 0.033 M KCl. After standing for 1½ to 3 h most of the supernatant was siphoned off, and the precipitate isolated by centrifugation in a Sorvall GSA* rotor at 4000 r.p.m. for 15 min. A volume of 1.2 M KCl equal to the weight of the precipitate was added to the latter, and the mixture stirred until the precipitate had dissolved. If necessary, the solution so obtained was clarified by centrifugation at 30,000 r.p.m. for 45 min in the Spinco Model L centrifuge. The solution was subjected to a second cycle of precipitation at an ionic strength of 0.033 and redissolution at 0.6. In all but two preparations bovine pancreatic ribonuclease at a concentration of 10 mg/ml was then added[4,5], 5 ml/l of myosin solution, yielding a ribonuclease concentration of 50 μg/ml. The solution was dialyzed against 0.6 M KCl for approximately 16 h, and subjected to a third cycle of precipitation and redissolution as above. Myosin prepared in this way was found to be free of myokinase activity.

Chromatography

In most cases, myosin obtained as described was, after dialysis against a suitable sample solvent, applied directly to a column of hydroxyapatite (Bio-Gel HT, obtained from Bio-Rad Laboratories, Richmond, Calif.) which had been pre-equilibrated with the same solvent. In a few cases the myosin was first subjected to chromatography on DEAE-Sephadex[6], and the major peak of the effluent, after concentration and dialysis, then applied to hydroxyapatite. DEAE-Sephadex[6] was preferred over DEAE-cellulose[1] because myosin is adsorbed more strongly to it, permitting the use of lower pH (7.5) and higher ionic strengths (0.4 to 0.6). Effluent fractions from hydroxyapatite were sometimes rechromatographed on a column of the same adsorbent. All chromatography was performed in jacketed Pharmacia columns 2.54 cm in diameter. Column length varied (see figures), but was usually approximately 32 cm.

Most column effluents were passed through a micro flow cell, maintained at 2–5°, in the cell compartment of a Beckman double-beam, grating spectrophotometer

* Mention of commercial names does not imply endorsement by the U.S. Department of Agriculture.

equipped with a programmer and a recorder. To prevent condensation on the cell, the compartment was flushed with dry nitrogen. The system was operated in the "point program" mode, and set to monitor and record transmittance at 259, 279, and 330 nm. The transmittance mode was preferred to the absorbance mode since the latter is limited to a range of one absorbance unit, while the range of the former is limited only by factors such as stray light and electronic noise. With the spectrophotometer used the effective transmittance range corresponds to 0–2.5 absorbance units. The slow monitoring cycle was used, with a period of approximately 2 ½ min.

After being monitored the effluent was collected by a refrigerated Buchler fraction collector, using a photoelectric volumetric collection head equipped with an auxiliary device ensuring its unfailing operation[7]. The photoconductive cell was set at a level which yielded tube fractions of 10 ml volume, the tubes being partially immersed in a liquid coolant maintained at 2–5°. Tube fractions which, on the basis of the recorder chromatogram, contained protein belonging to the same peak were pooled, yielding a "fraction".

Transmittance readings selected from the monitor record at suitably spaced intervals were each multiplied by the factor $100/T_0$, T_0 being the recorded transmittance (%) of the solvent at the corresponding wavelength, and converted to absorbance. The absorbance values of the pooled fractions were determined directly with a Beckman DU spectrophotometer calibrated for wavelength and absorbance[8]. Absorbance values were corrected for scattered light[9] according to the following formulae:

$$A'_{259} = A_{259} - \left(\frac{330}{259}\right)^2 A_{330} \tag{1}$$

$$A'_{279} = A_{279} - \left(\frac{330}{279}\right)^2 A_{330} \tag{2}$$

where A = measured absorbance, A' = corrected absorbance, and subscripts are the wavelength values in nm. From these corrected absorbance values, concentration of protein was calculated with simultaneous equations utilizing the absorptivities of myosin and nucleic acid at 259 and 279 nm[9–11]. These calculations were programmed and run on an Olivetti Programma 101. The chromatographic patterns were drawn with an IBM plotter. All recoveries reported are based on measurements made on pooled samples.

Assays for enzymatic activities

Myokinase was assayed by the method described by COLOWICK[12]. The concentration of KCl was kept minimal ($\leqslant$ 0.01 M), since high concentrations of salt inactivate this enzyme. Adenylate deaminase was assayed by a minor modification of the method described by NIKIFORUK AND COLOWICK[13]. The assay mixture was: 0.6 M in KCl; 0.05 M in citrate, pH 6.5; 80 μM in bicarbonate; 40 μM in AMP; and approximately 0.8 mg/ml in protein. Phosphate was removed from the protein by dialysis before assay. ATPase was determined as previously described[14].

RESULTS

A preliminary experiment showed that, at pH 6.8 and in the presence of 0.4 M KCl, no protein is eluted from hydroxyapatite until the orthophosphate concentration exceeds 0.1 M. On this basis a gradient elution system was chosen, with a constant KCl concentration of 0.4 M, a pH of 6.8, an initial phosphate concentration of 0.1 M, and a final phosphate concentration of 0.3 M. Fig. 1 shows the result of a run of this kind. A trace of protein appearing at a volume of approximately 50 ml was followed by a "doublet" at approximately 100 ml, and an additional small peak at approximately 150 ml, after which the major peak was eluted. Elution of the major peak was completed with the upper-limit buffer (0.4 M KCl, 0.3 M phosphate, pH 6.8),

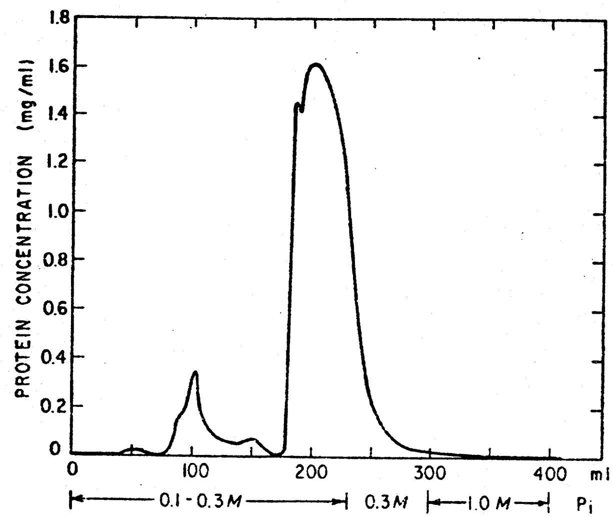

Fig. 1. Chromatography of myosin with a linear gradient of phosphate concentration. 100 mg of protein in 0.4 M KCl containing 1 mM phosphate were applied to a column of hydroxyapatite, 2.54 cm (diameter) × 18 cm (length), which had been equilibrated with the same solvent. P_i = phosphate. The concentration of KCl was constant at 0.4 M, except in the last elution solvent, which contained none. pH = 6.8 throughout. Recovery of protein: 88 %.

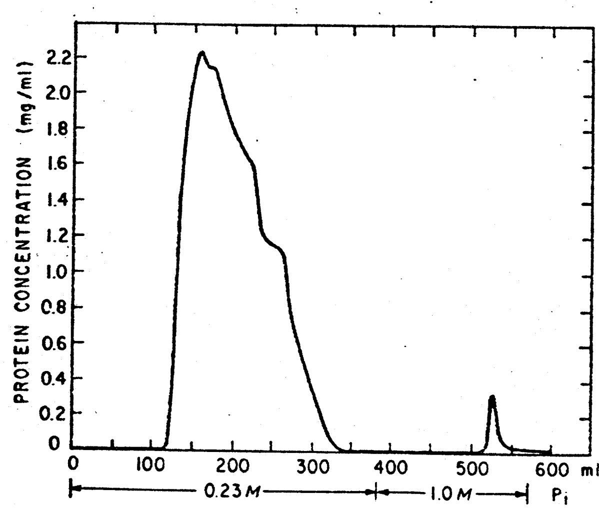

Fig. 2. Chromatography of myosin with a two-step elution program. 304 mg of protein were applied to a column, 34 cm long, which had been equilibrated with the first elution buffer. The first eluant was 0.4 M in KCl, the second had none. pH = 6.8. Recovery of protein: 63 %

after which the column was eluted with 1 M phosphate buffer, pH 6.8 (without KCl). In the instance shown the last buffer eluted no protein, but in most cases some protein was washed out of the column by this buffer (or by 0.4 M phosphate, when this was used; see below).

On the basis of the above gradient run (Fig. 1), and three others like it, a phosphate concentration of 0.23 M was chosen, for the purpose of stepwise elution, as just adequate to elute the major peak protein. A minimal concentration was chosen, rather than one on the high side of the peak, in order to permit resolution of the different components which may comprise the major peak (of the gradient runs). A typical chromatogram for such a run is shown in Fig. 2. Immediately apparent is the absence of the doublet, which, in the gradient run, preceded the major peak, as well as much earlier elution of the major peak (when the ratio of lengths of the columns in Figs. 1 and 2 is considered). The eluant containing 0.23 M phosphate (+ 0.4 M KCl) moves the major peak into the doublet, so that the latter is buried in the former, and no longer resolved from it. The major peak displays more character than in the gradient runs, indicating heterogeneity of the moysin preparation, but the components within the major peak are not resolved. As usual, 1 M phosphate elutes a small amount of protein.

To test whether different portions of the major peak (Fig. 2) contain different species of protein, it was divided into three portions; each portion was concentrated, and, after dialysis against the initial buffer, applied to a column of hydroxyapatite for re-chromatography (Fig. 3). Several runs of this kind showed no clearly demonstrable differences in elution volume for the different cuts of the major peak. Surprisingly, however, although all of the protein applied to the second column was in the major peak effluent of the first column, some protein was retained by the second column until it was eluted with 1 M phosphate, probably indicating conversion of major peak protein (isolated from the first column) into "1 M protein".

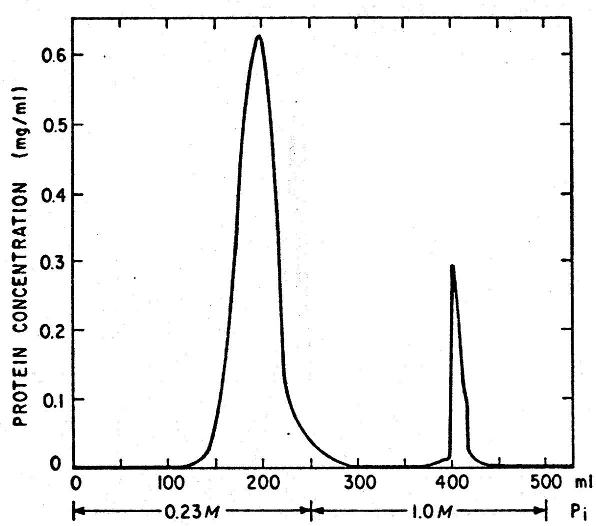

Fig. 3. Rechromatography of myosin on hydroxyapatite. 32 mg of protein, the first third of the fraction eluted by 0.23 M phosphate (+ 0.4 M KCl) from the column of Fig. 2, were applied to a column, 33 cm long. Other conditions as in Fig. 2. Recovery of protein: 88%.

The converse experiment was also run, *viz.*, protein collected in the 1 M effluent of the first hydroxyapatite column was, after concentration and dialysis, rechromatographed on a second column of hydroxyapatite (Fig. 4). In addition to the protein eluted in the expected (1 M) position, a substantial amount of protein was eluted elsewhere, *viz.* with the initial solvent, again indicating interconversion of the different proteins eluted by the 0.23 M and 1 M solvents. In these runs it was also noted that the amount of "1 M protein" appeared to increase with the post mortem age of the sample.

To effect resolution of the different components which might be present in

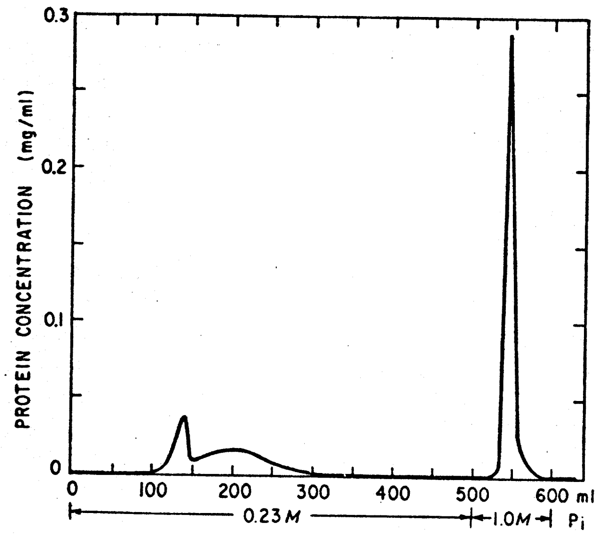

Fig. 4. Rechromatography of protein eluted by 1 M phosphate. 12 mg of protein eluted from the first column by 1 M phosphate were applied to the second column, 31 cm long. Other conditions as in Fig. 2. Recovery of protein: 100%.

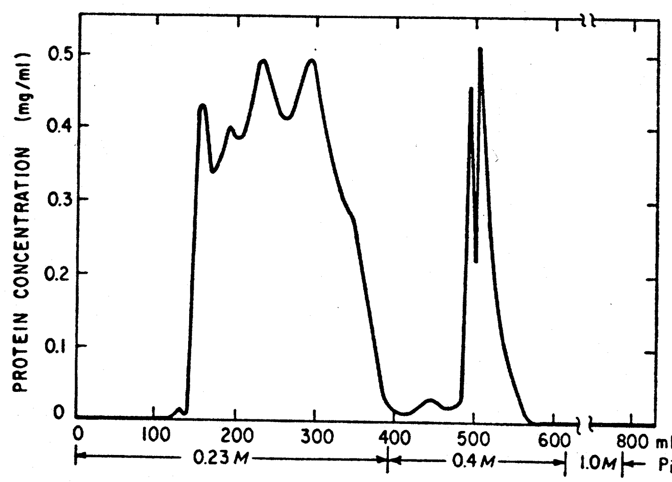

Fig. 5. Chromatography of myosin with a three-step elution program. 98 mg of protein obtained from the major peak effluent of a column of DEAE-Sephadex[6] was applied to a column of hydroxyapatite, 26 cm long. The first two eluant buffers contained 0.4 M KCl. Recovery of protein: 95%.

the 1 M phosphate effluent, the initial elution with 0.23 M phosphate (Fig. 2) was, in some runs, followed with 0.4 M phosphate (+ 0.4 M KCl) (Fig. 5.). Subsequent elution with 1 M phosphate did not bring off any protein. Apparently, all of the protein previously eluted with the 1 M solvent was now eluted with the 0.4 M phosphate solvent. However, the latter solvent achieved partial resolution of the effluent protein into two components. In the chromatogram of Fig. 5, the input protein consisted of the major peak obtained by chromatography on DEAE-Sephadex[6]. Preliminary chromatography on the latter absorbent may be a necessary condition for resolution in the 0.4 M phosphate solvent, but otherwise appears to have no discernible effect on the hydroxyapatite chromatogram.

Since, on initial elution of hydroxyapatite with 0.23 M phosphate (+ 0.4 M KCl), resolution of the "doublet" from the main peak was lost, the gradient chromatograms (in which the doublet was resolved) were reexamined. From these chromatograms it was estimated that a phosphate concentration of 0.16 M might effect separation of the doublet from the major peak. On this basis a four-step elution system was instituted (Fig. 6) with buffers of pH 6.8 containing phosphate at concentrations of 0.16, 0.23, 0.40, and 1 M. The first three buffers contained KCl at a level of 0.4 M; the last had no KCl. (The proteins eluted by these four buffers are referred to as fractions A, B, C, and D, respectively.) By means of this system the protein of the major peak was retarded, and the doublet was, indeed, clearly resolved from it.

The interconversion of protein between the fractions eluted by phosphate at low (0.23 M) and high ($\geqslant$ 0.4 M) concentrations suggested that the latter (fraction C or D) might be a polymer or a mixture of polymers of the former (fraction B). To test this hypothesis, the two effluents were subjected to sedimentation velocity analysis after concentration and dialysis against 0.23 M phosphate + 0.4 M KCl, pH 6.8. As shown in Fig. 7, fraction B protein sedimented as a typical, self-sharpening myosin peak, with a small amount of faster-running mateiral, while fraction C protein, run at the same concentration, yielded three peaks, none of which was

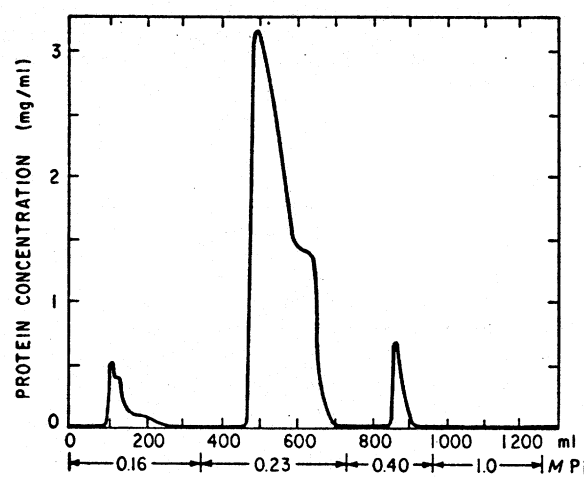

Fig. 6. Chromatography of myosin with a four-step elution program. 376 mg of protein were applied to a column, 33 cm long. The first three buffers contained KCl at a concentration of 0.4 M. Recovery of protein: 63%.

self-sharpening, and two of which sedimented more rapidly than the self-sharpening component of fraction B. Such a multi-peak pattern has been obtained frequently with aged myosin, and has been interpreted as resulting from "transformation reactions" of myosin[15,16].

— A further example of the consistency observed in the chromatograms obtained is shown in Table I, where the elution volumes are given for two runs on the same preparation; the second run was made seven days after the first. In spite of large differences in the amount of protein applied, in the input volume, and in the age of the samples, the elution volumes are in good agreement.

Adenylate deaminase content was determined, in one run, on three cuts from the major peak (fraction B) obtained as effluent from hydroxyapatite. Two of these

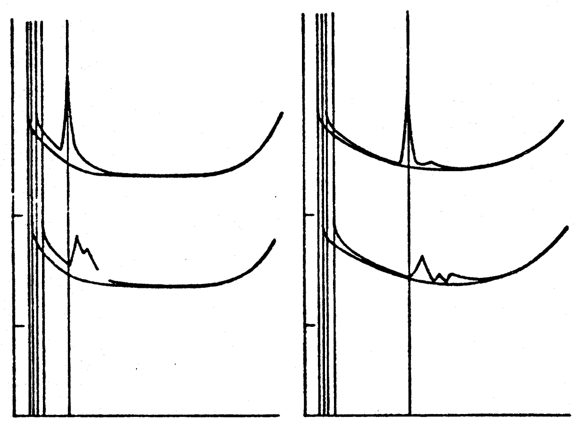

Fig. 7. Sedimentation velocity of the fractions eluted at low and high concentrations of phosphate. The samples were run at 25,980 r.p.m. for 50 min, then at 59,780 r.p.m. for the following time intervals: (a) 29 min and (b) 89 min. Wedge cell: fraction B, eluted by 0.23 M phosphate. Standard cell: fraction C, eluted by 0.4 M phosphate. Double-sector cells were used, with charcoal-Epon centerpieces and sapphire windows. Phase plate angle: 65°. Temperature: 4°. Protein concentration: 2.68 mg/ml in both cells.

TABLE I

REPRODUCIBILITY OF ELUTION VOLUME

Protein fraction	*Phosphate concn. (M) in eluant*	*Elution volume (ml) for myosin of age*[a]	
		5 Days	*12 Days*
A	0.16	130–170	100–170
B	0.23	150–310	140–340
C	0.40	140–180	130–190
Protein applied (mg)		101	376
Volume applied (ml)		6.2	22

[a] Post mortem age.

had a specific activity of 0.003, the third 0.008, μmole AMP/min/mg. The protein applied to the column had a specific activity of 0.049.

DISCUSSION

Of the twenty-five runs made on hydroxyapatite columns, the six which yielded the best recovery of protein were with myosin (input) samples which had previously been purified chromatographically, either on DEAE-Sephadex or on a previous column of hydroxyapatite. However, two input samples which had "seen" hydroxyapatite yielded only a moderate recovery, and one a low recovery. The probable reason for these results is apparent when recovery is plotted as a function of the (post mortem) age of the protein (Fig. 8). The recovery for the myosin previously purified with hydroxyapatite is distinctly higher than for "unpurified" myosin of the same age. For three samples (not plotted) which had been run on DEAE-Sephadex the recoveries were also high, *viz.* 91–100 %; these samples were 12–14 days old. It thus appears that, in addition to the three fractions (A, B, and C or D) into which myosin preparations are resolved by hydroxyapatite, there is a fourth fraction which is irreversibly adsorbed to this medium. Further elution with two column volumes of 1.6 M phosphate buffer at pH 6.8 and temperature 3° followed by two column volumes of KH_2PO_4 of the same molarity does not elute any detectable protein whatever. This fraction is also eliminated by chromatography on DEAE-Sephadex. Its amount depends on the age of the preparation. We have little quantitative data on the distribution of ATPase activity in the effluent peaks, but it is clear that the recovery of activity is quite low. Qualitatively, it can be stated that the doublet (fraction A) contains no ATPase, while both the major fraction (B) and the last fraction eluted (C or D) have ATPase activity. This is consistent with the interpretation that the latter fraction is a mixture of polymers of the former fraction.

The low recovery of enzyme may be due to denaturation of the myosin resulting from its low concentration in the effluents, or to the removal by hydroxyapatite

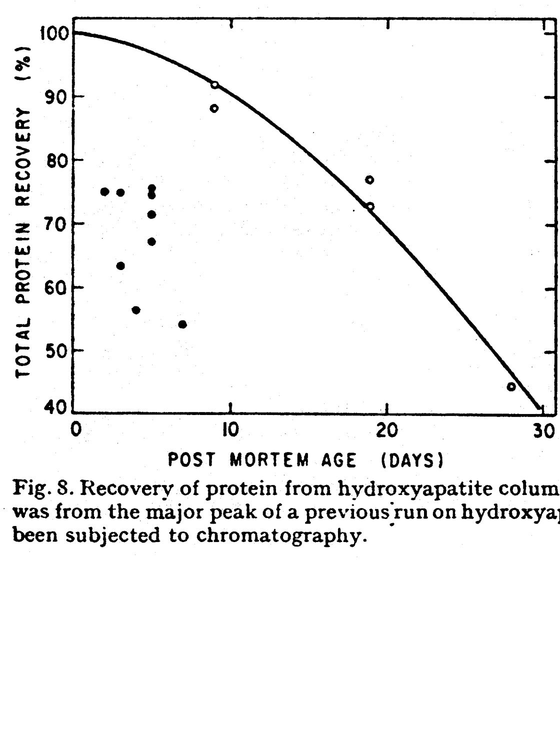

Fig. 8. Recovery of protein from hydroxyapatite columns as a function of age. ○, Sample applied was from the major peak of a previous run on hydroxyapatite; ●, column input had not previously been subjected to chromatography.

of nucleic acids or proteins which stabilize or regulate ATPase activity. The removal of proteins of low molecular weight closely associated with myosin is known to result in loss of enzymatic activity of myosin from white or fast muscle[17]. Most of the work described here was done with the longissimus dorsi muscle of the rabbit.

Data so far obtained have not demonstrated any substantial differences between the ribonuclease-treated myosins of "red" and "white" muscles of the rabbit. A more detailed comparison is being made.

In contrast to the appearance of fraction C (or D) on rechromatography on hydroxyapatite of the major fraction (B), fraction A does not reappear on rechromatography. Furthermore, although some fraction A is present even in myosin which has been purified by chromatography on cellulose phosphate and DEAE-Sephadex[6,10], the amount of A appears to be considerably less than in the unchromatographed preparation (unpublished experiments). Therefore, chromatography of a preparation of myosin on hydroxyapatite yields clear-cut information on contamination, *i.e.* the presence of proteins other than myosin (peak A), as well as on the presence of aggregated myosin (peak C). Comparable data have not been obtained by any other means.

Chromatography on DEAE-cellulose yields chromatograms in which there is a doubled peak of specific ATPase activity (Fig. 5 in Ref. 1). This could conceivably result from resolution of two enzymatically active protein species, as discussed by PERRY[1]. However, when these two effluent fractions were isolated and rechromatographed, the positions of the effluent peaks overlapped, the peak maxima apparently being approximately 10 ml apart (Fig. 2 in Ref. 1). The alternative explanation, offered by PERRY as a possibility, *viz.* that there is enzymatically inactive material in the middle of the myosin ATPase peak, therefore appears by far the more plausible. Thus, the patterns obtained with DEAE-cellulose, while suggesting heterogeneity, suffer from a failure to satisfactorily resolve the components responsible for the heterogeneity. By contrast, the fractions obtained with hydroxyapatite are completely resolved. Thus (Fig. 6), peaks A and B are separated by more than 200 ml and B and C by more than 150 ml.

REFERENCES

1 S. V. PERRY, *Biochem. J.*, 74 (1960) 94.
2 J. E. GODFREY AND W. F. HARRINGTON, *Biochemistry*, 9 (1970) 886.
3 D. W. QUASS AND E. J. BRISKEY, *J. Food Sci.*, 33 (1968) 180.
4 E. MIHALYI, K. LAKI AND M. I. KNOLLER, *Arch. Biochem. Biophys.*, 68 (1957) 130.
5 E. F. BARIL, D. S. LOVE AND H. HERMANN, *J. Biol. Chem.*, 241 (1966) 822.
6 E. G. RICHARDS, C.-S. CHUNG, D. B. MENZEL AND H. S. OLCOTT, *Biochemistry*, 6 (1967) 528.
7 J. A. CONNELLY AND S. M. MOZERSKY, *Anal. Biochem.*, 35 (1970) 537.
8 G. W. HAUPT, *J. Opt. Soc. Amer.*, 42 (1952) 441.
9 E. MIHALYI AND A. J. ROWE, *Biochem. Z.*, 345 (1966) 267.
10 M. HARRIS AND C. H. SUELTER, *Biochim. Biophys. Acta*, 133 (1967) 393.
11 O. WARBURG AND W. CHRISTIAN, *Biochem Z.*, 310 (1941) 384.
12 S. P. COLOWICK, *Methods Enzymol.*, 2 (1955) 598.
13 G. NIKIFORUK AND S. P. COLOWICK, *Methods Enzymol.*, 2 (1955) 469.
14 S. M. MOZERSKY, J. D. PETTINATI AND S. D. KOLMAN, *Anal. Chem.*, 40 (1968) 788.
15 P. JOHNSON AND A. J. ROWE, *Biochem. J.*, 74 (1960) 432.
16 P. JOHNSON AND A. J. ROWE, *Biochim. Biophys. Acta*, 53 (1961) 343.
17 F. J. SAMAHA, L. GUTH AND R. W. ALBERS, *J. Biol. Chem.*, 245 (1970) 219.